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Hepatitis B Virus Surface (S) Transactivator With DNA-Binding Properties

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Chronic infection with hepatitis B virus (HBV) in humans is strongly linked to the development of hepatocellular carcinoma (HCC). Activation of growth-regulatory genes may play a crucial role in carcinogenesis. Proto-oncogene expression has been shown to be higher in HCC tissue with integrated HBV DNA than in the normal liver. Earlier, we showed that the 3′ end of the HBV major surface gene (S) (426-855 nucleotides of the S region) is a transactivator of the X promoter–enhancer regulatory element in co-transfection experiments. This region expresses a truncated carboxy terminal S protein extending from amino acid residues 102 to 226. In this study, the truncated S protein (trc-S) was examined for its enhancing activity on several viral and cellular regulatory elements. The results indicate that trc-S activates rous sarcoma virus long terminal repeat (LTR), human T-lymphotropic virus 2 LTR, human immunodeficiency virus 1 LTR, and the c-*jun* and c-*fos* promoters. Electrophoretic mobility shift assays carried out to investigate its DNA-binding properties established that trc-S binds to HBV X promoter and oligonucleotides representing binding sites for the AP1 and TFIID transcription factors. The specificity of this interaction was confirmed by using competition experiments and supershift assays. These experiments suggest that trc-S is a transactivator of several cellular and viral promoters and that this activity is mediated by direct interaction with DNA. ***J. Med. Virol. 61:1–10, 2000.***

KEY WORDS: hepatitis B virus; transactivator; hepatocellular carcinoma; cellular and viral promoters; electrophoretic mobility shift assay

INTRODUCTION

Hepatitis B virus (HBV) causes a wide spectrum of liver diseases, ranging from acute to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). About 350 million people worldwide are infected chronically with HBV [Beasely and Hwang, 1984]. These individuals have a greater than hundredfold higher predisposition to HCC. HCC is a highly malignant tumour with a very poor prognosis and ranks as the seventh most common cancer of human beings.

HBV is a DNA virus with a circular, partially double-stranded genome approximately 3.2 kb in length. The genome comprises four open reading frames: surface, core, polymerase, and X. These open reading frames code for various viral polypeptides. In recent years, molecular biologic studies of HBV and prospective investigations of patients with cirrhosis, who are at risk for cancer, have considerably advanced our knowledge of the pathogenesis and natural course of HCC. Various events leading to neoplastic transformation are still not completely understood. Insertional mutagenesis and transactivation by viral gene products have been suggested as possible mechanisms of HBV-associated cell transformation. These actions lead to altered expression of oncogenes and/or growth-regulatory genes. HBV DNA integrates into the host genome in a sequence-nonspecific manner [Yaginuma et al., 1987; Shih et al., 1988]. By acting as an insertional mutagen, HBV has been shown to alter the activity of proto-oncogenes, such as c-*myc* [Wei et al., 1992], c-*fos*, and c-*jun* [Varmus, 1984]; the tumour supressor gene p53 [Bressac et al., 1990]; and cellular genes for cyclin A [Wang et al., 1990] and retinoic acid receptor [de The et al., 1987].

On the other hand, several subgenomic fragments of HBV, for example, the X protein [Zahm et al., 1988; Colgrove et al., 1989], pre-S2/S [Caselmann et al., 1990], and pre-S1 [Kim et al., 1997], have been shown to be potential transactivators. In the case of the X gene, a clear association with c-*myc* expression has

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been verified [Terradillos et al., 1997]. In an earlier study, it was found that the 3′ end of a major surface gene, covering nucleotides 636 to 746, is most frequently identified in HCC tissue [Ramesh et al., 1994]. The potential of this region to transactivate the HBV X promoter–enhancer cascade was also demonstrated. The present study further characterizes further this truncated S transactivator (trc-S) in terms of its activity on other viral and cellular regulatory elements and shows that this activity possibly is mediated by direct interaction of trc-S with genetic regulatory elements.

MATERIALS AND METHODS

Expression of Truncated S Transactivator in *Escherichia Coli*

Plasmid pSG41 [Ramesh et al., 1994], which has an HBV S region, nucleotides 426-855 cloned into eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA), was used to investigate transactivation function and DNA–protein interactions. Truncated S transactivator extending from nucleotide 426 to nucleotide 855 was cloned in-frame, using prokaryotic expression vector pGEX-4T-2 (Pharmacia Biotech, Uppsala, Sweden). In this vector, the recombinant proteins are expressed as fusion proteins with glutathione-*S*-transferase gene (*GST*). The expression of recombinant protein was obtained by induction with 1 mmol/L isopropylthiogalactoside (IPTG) (Sigma Chemical Co., St. Louis, MO) in the presence of 2% glucose at 30°C for 7 hours and visualised by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Immunodetection of the recombinant S transactivator protein induced in *E. coli* was carried out after transfer of electrophoresed proteins to the nitrocellulose membrane. Polyclonal anti–hepatitis B surface antigen (anti-HBsAg) was used in a 1:500 dilution for Western blot analysis. Horseradish peroxidase–conjugated antibody (Dakopatts, Denmark) raised in Rabbit was used as a secondary antibody at a 1:1,000 dilution. The blot was developed with diaminobenzedine (Sigma).

Cell Culture and Transfection

Plasmid pSG41 [Ramesh et al., 1994] was used for all transactivation analyses. HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Bethesda, MD) supplemented with 10% (vol/vol) fetal bovine serum (Life Technologies) in a 5% CO_2 atmosphere. Cells were seeded at a density of 3×10^6 in 60-mm plates 1 day before transfection. DNA was transfected into the cells using Lipofectamine (Life Technologies) according to the manufacturer's instructions. The cells were analyzed 48 hours post-transfection.

Plasmid pSG41 was co-transfected with plasmids containing different viral and cellular promoters regulating the chloramphenicol acetyltransferase (*CAT*) reporter gene. The ratio of pSG41 to reporter was kept at 2:1 or 3:1 in all co-transfection assays. The promoter plasmids used in the study were pRSV-CAT with the long terminal repeat (LTR) of Rous sarcoma virus [Gorman et al., 1982a]; pHTLV type 1– and pHTLV type 2–CAT with the LTR of human T-lymphotropic virus 1 (HTLV-1) or 2 (HTLV-2), respectively [Sodroski et al., 1984]; pHIV type 1–CAT with the LTR of human immunodeficiency virus 1 (HIV-1) [Seto et al., 1988]; pCP-CAT with the HBV core promoter and enhancer [Jameel and Siddiqui, 1986]; pSV2-CAT with the SV40 early promoter–enhancer [Gorman et al., 1982b]; and c-*fos*- and c-*jun*-CAT with the c-*fos* [Deschamps et al., 1985] and c-*jun* [Angel et al., 1988] promoters. Plasmid pCH110 carrying a β-galactosidase reporter gene, driven by the SV40 early promoter–enhancer (LKB Pharmacia, Sweden), was used as an internal control for transfection efficiency; pSG5 (mock) transfected and nontransfected HepG2 cells were used as negative controls.

Chloramphenicol Acetyltransferase Assay

CAT assay was performed essentially as described by Gorman et al. [1982b] using [^{14}C]chloramphenicol (Amersham, England). Cells were harvested, washed twice in phosphate-buffered saline, suspended in 0.25 mol/L Tris-HCl (pH 7.8), and disrupted by three cycles of freezing and thawing. The protein concentration was estimated using the Bradford assay (Bio-Rad, Hercules, CA). CAT activity was assayed in samples having the same amount of β-galactosidase activity in an individual experiment. Each experiment was repeated a minimum of three times. CAT activity was quantitated by excising spots corresponding to the acetylated forms from thin-layer chromatography (TLC) plates, and the radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

Oligonucleotides

Oligonucleotides were designed using the OLIGO 4.0 software and synthesised on an automated DNA synthesiser (model 392; Applied Biosystems, Foster City, CA) using phosphoramidite chemistry. Primers were designed to amplify HBV X (nucleotides1242-1365) and core (nucleotides 1686-1849) promoters, respectively (Table I). The sense and antisense oligonucleotides representing the consensus binding sites of AP1 and TFIID were also synthesised (Table I). Before use, oligonucleotides were purified by electrophoresis on an 8 mol/L urea, 15% polyacrylamide gel.

In Vitro Translation

For electrophoretic mobility shift assays, trc-S was produced in vitro using a coupled transcription–translation system (Amersham, England) primed with plasmid pSG41 according to the manufacturer's guidelines.

Probe Preparation

Primer pairs 1245/1367 or 1689/1852 (Table I) were used to amplify the HBV X and core promoters, respectively, by polymerase chain reaction (PCR) using HBV/pBS+ (3.2-kb whole HBV genome cloned into pBS+) as template. Initial denaturation at 95°C for 4 minutes

TABLE I. Sequences of Primers Used in This Study

X promoter	
Forward (nt. 1245–1264)	5′ GGAACCTTTGTGGCTCCTCT 3′[a]
Reverse (nt. 1348–1367)	5′ GTATTTCCGAGAGAGGACAA 3′[a]
Core promoter	
Forward (nt. 1689–1710)	5′ CAACGACCGACCTTGAGGCATA 3′[a]
Reverse (nt. 1831–1852)	5′ TGGAGGCTTGAACAGTAGGACA 3′[a]
AP1	
Forward	5′ GATCCCGCTTGATGAGTCAGCCGGAA 3′
Reverse	5′ GATCTTCCGGCTGACTCATCAAGCGG 3′
TFIID	
Forward	5′ GATCCGCAGAGCATATAAGGTGAGGTAGGAA 3′
Reverse	5′ GATCTTCCTACCTCACCTTATATGCTCTGCG 3′

[a]nt., nucleotide. Positions according to subtype adw2.

was followed by 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 8 minutes. The PCR products were labelled by incorporation of [α-^{32}P]dCTP (BARC, India) and were purified by Qiaquick PCR purification columns (Qiagen, Germany). Sense oligonucleotides of both AP1 and TF11D were labelled at the 5′ end by incorporation of [γ-^{32}P]dCTP (BARC), using T4 polynucleotide kinase (Promega, Madison, WI). Double-stranded oligonucleotides were obtained by annealing each labelled sense strand with the corresponding antisense strand in annealing buffer (0.05 mol/L NaCl, 6 mmol/L Tris-HCl at pH 7.5, 8 mmol/L $MgCl_2$) by heating for 5 minute at 68°C, followed by slow cooling at room temperature.

Preparation of Cell Extracts

HepG2 cells were transfected with pSG41 or with the pSG5 vector (mock-transfected); 48 hours later, nuclear and cytoplasmic extracts were prepared from 5–8 × 10^6 cells [Natoli et al., 1994]. Extracts were also prepared from nontransfected HepG2 cells. All steps for the preparation of the extracts were carried out at 4°C. Protein concentration of the extracts was determined with the Bradford protein assay (Bio-Rad).

Electrophoretic Mobility Shift Assays

The trc-S protein synthesized in vitro was used for binding assays. The ^{32}P-labeled core and X promoters, as well as the AP1 and TFIID binding sites, were used as substrate. Binding reactions were carried out in a total volume of 15 μl at room temperature for 30 minutes. The binding buffer contained 20 mmol Tris-HCl at pH 7.4, 50 mmol $MgCl_2$, 1 mmol/L EDTA, and 300 μg bovine serum albumin. Poly (dI-dC) (Amersham) was added in excess (2 μg) to the labelled probe (250 fmol, ~60,000 cpm). The reaction mix from a translation reaction, without any plasmid substrate, was used as a negative control. Specific competition reactions were carried out with 4–6 pmol of unlabelled probe. Electrophoresis was carried out on a 4% non-denaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 4°C. The gel was pre-run at 10 mA for 15 minutes. The samples were mixed with gel-loading dye (1× binding buffer, 20% glycerol, 1 mg/ml bromophenol blue), and electrophoresis was carried out at 30 mA until the dye front reached three-fourths of the gel length. The gel was wrapped in plastic wrap and exposed to X-ray film. The migration pattern was visualised by autoradiography.

Nuclear and cytoplasmic extracts prepared from pSG41-transfected, mock-transfected, or nontransfected HepG2 cells also were used for electrophoretic mobility shift assays, as described earlier herein. The nuclear extracts from cells transfected with 2–8 μg pSG41 were used for the binding reactions with the labelled probe (250 fmol, ~60,000 cpm). Competition reactions were carried out using either 250 fmol to 4 pmol or 1–4 μg of the specific unlabelled probe. A fragment of DNA from hepatitis E virus (nucleotides 7084-7194), generated by PCR amplification, was used as a nonspecific competitor.

Further specificity of the binding was confirmed by using polyclonal antibody to HBsAg in a supershift assay. For this purpose, in vitro translated trc-S was used. Briefly, 8 μl and 10 μl of polyclonal anti-HBsAg was added to the binding reaction (as described previously) following the initial 30-minute incubation. The reaction mix was incubated for another 30 minutes at 30°C before being loaded onto nondenaturing polyacrylamide gel. Human serum negative for anti-HBsAg was used as a negative control in these assays.

RESULTS

Expression of Truncated S Protein in Prokaryotic Cells

A recombinant fusion product, consisting of truncated HBV surface protein and GST protein, was expressed in *E. coli* BL21 (DE3) cells after induction with IPTG. The 41.6-kd fusion product with 26-kd GST protein at the N terminal was visualised on SDS-PAGE stained with Coomassie brilliant blue (Sigma) (Fig. 1a). On Western blot analysis, the immunoreactivity of this protein was established with anti-HBsAg (Fig. 1b).

Truncated S Protein Transactivation of Cellular and Viral Promoters

The ability of the 3′ end of the HBV S gene (nucleotides 426 to 855) product (trc-S) to activate different viral and cellular promoters was tested by co-transfecting HepG2 cells with plasmid pSG41 and CAT reporter plasmids carrying different promoters. On co-

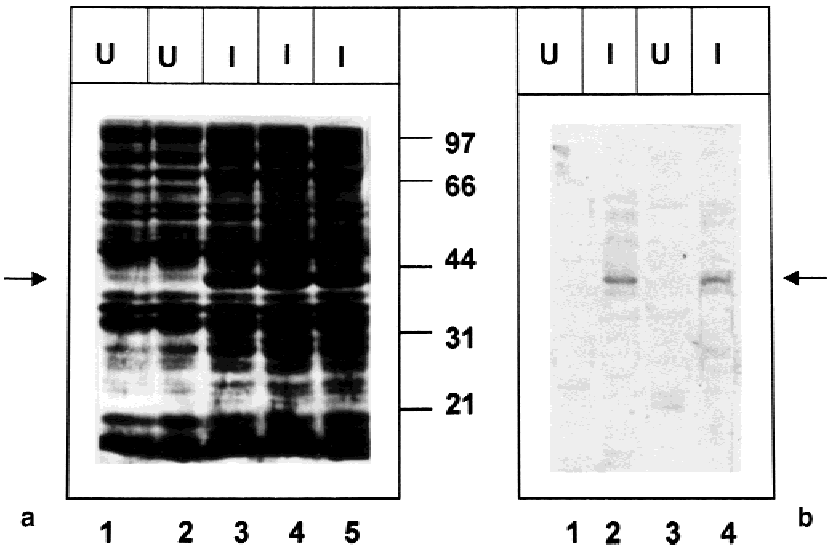

Fig. 1. **a:** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis showing expression of a 41.6-kd recombinant fusion protein, consisting of truncated carboxy terminal HBV surface protein (trc-S) and GST in *E. coli* BL21 DE3 cells. **Lanes U:** noninduced; **lanes I:** induced cells. **b:** Western blot analysis of the 41.6-kd recombinant fusion protein, induced in *E. coli* cells shows immunoreactivity with polyclonal anti-HBsAg antibodies (lanes 2 and 4).

transfection with pSG41, CAT expression from rous sarcoma virus (RSV) LTR, HTLV-2 LTR, HIV-1 LTR, and c-*jun* and c-*fos* promoters was found to increase. The enhancement of CAT activity over the basal level generated with reporter plasmid alone was used as a measure of transactivation.

The cellular promoters c-*jun* and c-*fos* showed five- and threefold increases in activity, respectively, by co-expression of trc-S (Figs. 2 and 3). The viral promoters HTLV-2 LTR and HIV-1 LTR showed a modest increase in CAT activity, corresponding to 2.6- and 2.2-fold, respectively, over the basal level (Fig. 4). With the RSV LTR, however, a slightly higher level of enhancement (4.3- fold) was shown (Fig. 4). No increase in CAT activity by the truncated S gene product was observed with HTLV-1 LTR, SV40 early promoter–enhancer, or HBV core promoter–enhancer *I* in HepG2 cells. Transfection assays with each promoter–reporter plasmid was carried out in triplicate. A decline in the enhancement of CAT activity was observed with the increase in the quantity of transactivator plasmid (pSG41) used in the experiment, at a constant amount of reporter plasmid (1 μg/plate) (Table II). This repression at higher concentrations was found consistently for RSV LTR, HTLV-2 LTR, HIV-1 LTR, and c-*jun* and c-*fos* promoters.

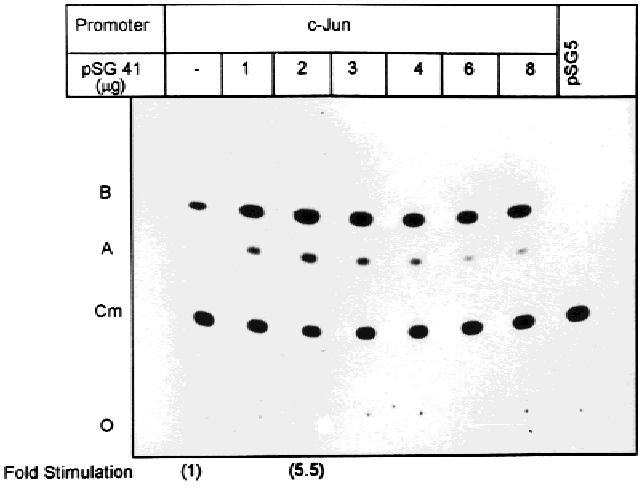

Fig. 2. Enhancement of chloramphenicol acetyltransferase (CAT) activity in HepG2 cells co-transfected with plasmid pSG41 and a CAT reporter plasmid driven by c-*jun* promoter. CAT activity was measured in cell lysate. The autoradiograph shows the variation in CAT activity at a fixed concentration of reporter plasmid to increasing concentrations of pSG41. The cell extracts prepared from pSG5-transfected cells were used as negative control. O, origin; Cm, nonreacted chloramphenicol: A, 1-acetylchloramphenicol; B, 3-acetylchloramphenicol.

Truncated S Protein Binding to the Promoter Elements

The ability of trc-S protein to bind different promoters was investigated by an electrophoretic mobility shift assay (EMSA). The DNA probes were radiolabelled, and the formation of stable DNA–protein complexes was visualised by autoradiography after polyacrylamide gel electrophoresis. Poly (dI-dC) was added in excess to all the reaction mixtures, to compete for nonspecific DNA-binding proteins. The effects of varying concentrations of trc-S on DNA–protein interactions were compared. To determine the specificity of the binding, competition experiments were carried out using varying concentrations of specific and nonspecific competitors. Further confirmation of the DNA–protein interaction comes from a supershift experiment.

The trc-S protein (~17 kd) produced in vitro was used to study its binding to HBV X and core promoters and to oligonucleotides representing the binding sites for AP1 and TFIID transcription factors. The HBV X promoter formed a single bound complex (SB) with in vitro expressed trc-S protein (Fig. 5a). There was a decrease in the intensity of the retarded band when 4–6 pmol of the specific unlabelled probe was used for competition (Fig. 5a, lanes 5 and 6). No binding with the X promoter was observed when the binding reaction was carried

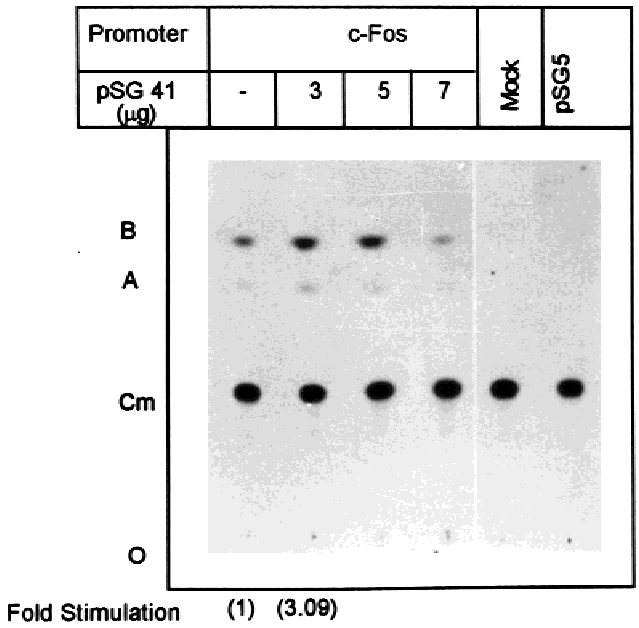

Fig. 3. Co-transfection of HepG2 cells with plasmid pSG41 and a chloramphenicol acetyltransferase (CAT) reporter plasmid driven by c-*fos* promoter. CAT activity was measured in cell lysate. The extracts prepared from pSG5-transfected cells were used as negative control. O, origin; Cm, nonreacted chloramphenicol: A, 1-acetylchloramphenicol; B, 3-acetylchloramphenicol.

out with the product from a control in vitro translation reaction, without any plasmid substrate (Fig. 5a, lane 7). The trc-S protein expressed in HepG2 cells also showed binding to the HBV X promoter. An SB was observed when the binding reactions were carried out between the HBV X promoter and nuclear extract prepared from pSG41-transfected HepG2 cells (Fig. 5b). The complex could be competed out using 1–4 μg specific unlabelled DNA probe (Fig. 5b, lanes 5 and 6). No specific complex formation was observed between the probe prepared from HBV X promoter and the nuclear extract prepared from mock-transfected HepG2 cells (Fig. 5b, lane 8). On increasing the amount of protein, an initial increase in the bound DNA was observed (Fig. 5b, lanes 2–4), which was followed by decreased binding (Fig. 5b, lane 7).

On binding the trc-S produced in vitro with the oligonucleotides representing the binding sites of TFIID and AP1, we observed the presence of two retarded bands (SB1, SB2) (Figs. 6a and 7a). A corresponding lower band (SB1) was also observed when the binding reaction of TFIID and AP1 oligonucleotides was carried out with the product from the in vitro control reaction (Fig. 6a, lane 9; Fig. 7a, lane 7). The binding reaction with the control was conspicuous by the complete absence of the second retarded band (SB2), which was seen only in the binding between trc-S and AP1 and TFIID oligonucleotides. These could be competed out using 1–4 pmol of the specific unlabelled competitor (Fig. 6a, lanes 3, 6, 7, and 8; Fig. 7a, lanes 3, 5, and 6). On using a nonspecific competitor, there was no decrease in the intensity of the shifted bands.

The DNA fragments, representing binding sites of TFIID and AP1, also showed two retarded bands (SB1, SB2) with the nuclear extract prepared from pSG41-transfected HepG2 cells (Figs. 6b and 7b). The lower retarded band, SB1, was also observed when the nuclear extract prepared from the mock (pSG5)–transfected HepG2 cells was used for binding with TFIID (Fig. 6b, lane 12) and AP1 binding domains (Fig. 7b, lane 12). When the DNA–protein complex was subjected to 0.1% SDS treatment, complete disruption of the complex was observed (Fig. 6b, lane 11). As earlier, there was an increase in the binding of DNA with the increase in the concentration of protein (Fig. 6b, lanes 2, 3, and 6; Fig. 7b, lanes 2–5). Peak binding was observed at 5.0 μg of the nuclear extract with 250 fmol of the oligonucleotide probe (Fig. 6b, lane 6; Fig. 7b, lane 5). On increasing the amount of protein concentration, there was a decline in the relative intensity of the retarded bands (Fig. 6b, lane 4; Fig. 7b, lane 8). Competition studies with unlabelled DNA provided further

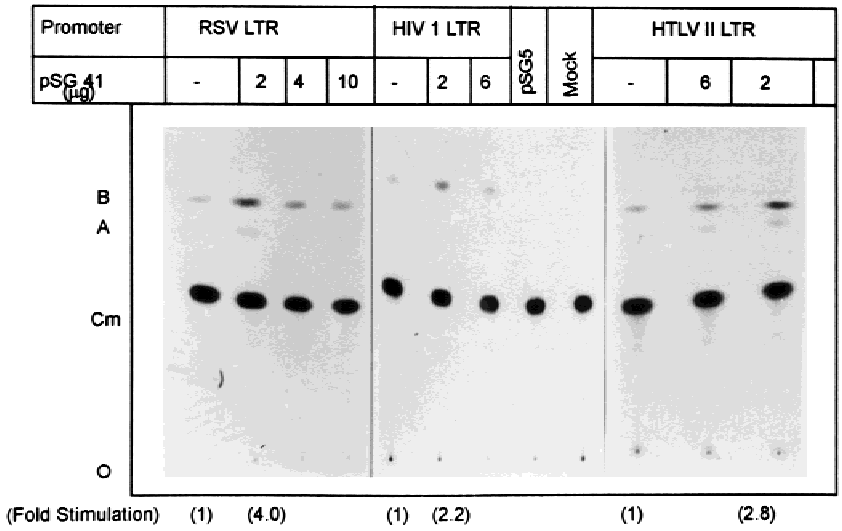

Fig. 4. Effect of truncated S protein on the expression of chloramphenicol acetyltransferase (*CAT*) gene regulated by rous sarcoma virus long terminal repeat (RSV LTR), human immunodeficiency virus 1 (HIV-1) LTR, and human T-lymphotropic virus 2 (HTLV-2) LTR sequences in HepG2 cells. Cell extract from pSG5 and nontransfected HepG2 cells (mock) indicate the controls. O, origin; Cm, nonreacted chloramphenicol: A, 1-acetylchloramphenicol; B, 3-acetylchloramphenicol. The number of fold stimulation CAT activity is given below.

TABLE II. Effects of Varying Quantities of pSG41 on Chloramphenicol Acetyltransferase (CAT) Activity When Co-transfected With a Fixed Amount of Reporter Plasmid in HepG2 Cells*

Name of the promoter	Concentration of pSG41 in μg (fold CAT activity)		
c-*jun*	2	3	4
	(5.06 ± 0.11);	(3.27 ± 0.3);	(2.4 ± 0.4)
c-*fos*	2	3	4
	(1.62 ± 0.3);	(3.0 ± 0.09);	(1.03 ± 0.15)
RSV LTR	2	4	10
	(4.33 ± 0.41);	(2.07 ± 0.11);	(1.43 ± 0.40)
HIV-1 LTR	2	4	6
	(2.2 ± 0.15);	(1.43 ± 0.25);	(1.1 ± 0.1)
HTLV-2 LTR	2	4	6
	(2.6 ± 0.20);	(1.16 ± 0.15);	(0.87 ± 0.11)

*RSV, rous sarcoma virus; LTR, long terminal repeat; HIV-1, human immunodeficiency virus 1; HTLV-2, human T-lymphotropic virus 2. Transfections were carried out in HepG2 cells, using 1 μg of each reporter plasmid. The ratio of acetylated chloramphenicol in co-transfection with pSG41 to acetylation with the reporter plasmid alone is shown as fold CAT activity. The numbers are the means ± SD of three separate transfections; pCH110 was used as an internal control of transfection efficiency.

evidence of binding specificity. With increasing amounts of unlabelled competitor (in the range of 500 fmol to 4 pmol), the intensity of the DNA–protein complex decreased (Fig. 6b, lanes 5, 7, 8, 9, and 10; Fig. 7b, lanes 6, 7, 9, 10, and 11). There was no decrease in the intensity of the retarded band when nonspecific unlabelled probe was used as competitor. The trc-S protein produced either in vitro or in vivo did not show any binding to the HBV core promoter. The cytoplasmic extract prepared from pSG41-transfected or mock-transfected HepG2 cells did not show any binding with X promoter or AP1 and TFIID oligonucleotides.

To confirm binding specificity, supershift assays were carried out with polyclonal antiserum against HBsAg and in vitro–produced trc-S. Addition of anti-HBsAg antibody to the reactions caused a reduction in the intensity of DNA–protein complex (SB2) (Fig. 8, lanes 3 and 4). In addition, a new complex with further retardation in mobility was observed (Fig. 8, lanes 3 and 4). Appearance of the retarded complex also correlated with the amount of antibody added, in that more antibodies increased the intensity of the slower-moving complex and diminished the intensity of the complex SB2. These findings were observed for both TFIID

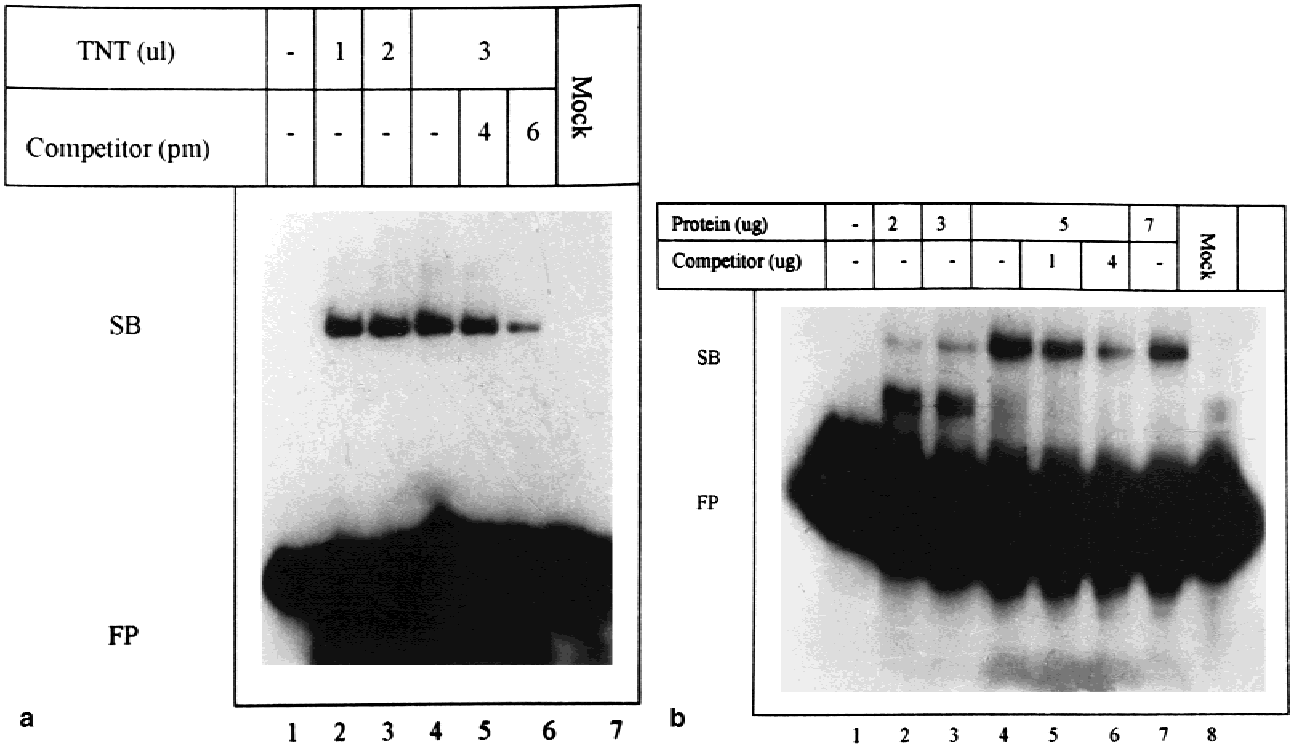

Fig. 5. **a:** Electrophoretic mobility shift assay performed with radiolabelled HBV X promoter (1245-1367) and in vitro synthesized truncated S protein (lanes 2–4). The specificity of the shifted band was confirmed by competition assays (lanes 5 and 6). The amount of specific competitor used is indicated in the figure. The mock lane 7 indicates the binding of HBV X promoter with the product from a control in vitro translation reaction, without the presence of plasmid substrate. FP, free probe (**lane 1**); SB, shifted band; TNT, transcription-linked translation lysate. **b:** Electrophoretic mobility shift assay shows the interaction between radiolabelled HBV X promoter (1245-1367) and nuclear extract prepared from pSG41-transfected HepG2 cells (**lanes 2, 3, 4, and 7**). Binding assays were performed as described in the text. Labelled probe in the amount of 250 fmol, ~60,000 cpm, was used. The amount of specific competitor is indicated at the top (**lanes 5 and 6**). Binding with the nuclear extract prepared from pSG5 (vector)–transfected HepG2 represents the negative control (lane 8). FP, free probe (lane 1); SB, shifted band.

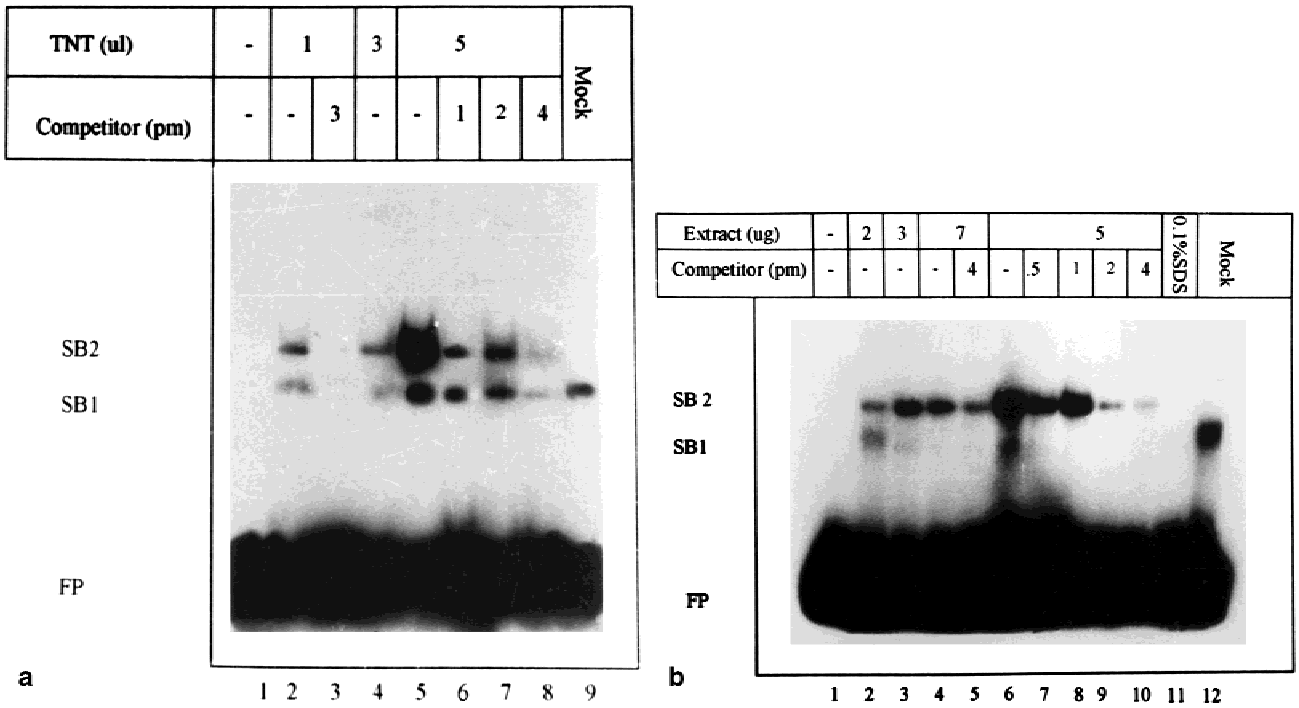

Fig. 6. **a:** Gel retardation analysis showing the interaction between oligonucleotide representing the TFIID binding site and in vitro–synthesised truncated S protein (**lanes 2, 4, and 5**). The specificity of the shifted band was confirmed by competition assays (**lanes 3, 6, 7, and 8**). The amount of specific competitor is indicated at the top of the lane. The mock lane (lane 9) indicates control binding with the non-translated transcription-linked translation (TNT) lysate. FP, free probe (**lane 1**); SB1, shifted band 1; SB2, shifted band 2. **b:** Electrophoretic mobility shift assay showing the interaction between radiolabelled TFIID binding sites and nuclear extracts prepared from pSG41-transfected HepG2 cells (**lanes 2, 3, 4, and 6**). Binding assays were performed as described in the text. Labelled probe in the amount of 250 fmol, ~60,000 cpm, was used. The amount of specific competitor is indicated at the top of the lane (**lanes 5, 7, 8, 9, and 10**). Binding with the nuclear extract prepared from pSG5 (vector)–transfected HepG2 represents the mock (**lane 12**). FP, free probe (**lane 1**); SB1, shifted band 1; SB2, shifted band 2. (**Lane 11** shows disruption of the complex by .1% SDS.)

binding sites (Fig. 8) and AP1 (data not shown). There was no interaction between the DNA–protein complex and human serum negative for anti-HBs (Fig. 8, lane 5).

DISCUSSION

The exact mechanism of action by which HBV causes neoplastic transformation of hepatocytes is not known. In chronic liver disease, HBV core antigen expression and the associated cytotoxic T-cell response leads to inflammation and liver cell necrosis [Chisari et al., 1989]. In the background of this chronic inflammation, integrated viral genome in the host cell can modify the expression of growth-regulatory genes. Thus, in a liver with continuous cell regeneration, it is possible that altered growth can lead to cellular transformation. Earlier it was shown that 91% of HBV-associated HCC tissue was positive for the 3′ end of the S gene (nucleotides 636 to 746) [Ramesh et al., 1994]. The transactivators X and pre-S2/S were present only in 31.8% and 54.5% of cases, respectively. The transactivation potential of this frequently detected truncated HBV S fragment was shown using the X promoter–enhancer *I*-CAT reporter system. The subgenomic fragment used, nucleotides 426-855, covers the 3′ end of the major surface gene (*S*) of HBV and represents a truncated carboxy terminal S protein (trc-S) extending from amino acid residues 102 to 226.

The present study shows that the S-transactivator region is an activator of several viral and cellular promoters. These investigations used transient expression of the bacterial *CAT* gene, under the control of potential target promoters in transfected HepG2 cells. The truncated carboxy S transactivator was provided in trans by co-transfecting with a second plasmid containing the S fragment under control of the SV40 early promoter–enhancer (pSG41). Plasmid pCH110 containing β-galactosidase expression driven by SV40 early promoter and enhancer was used as an internal control of transfection efficiency.

In such co-transfection experiments, trc-S was found to activate the RSV LTR, HTLV-2 LTR, and HIV-1 LTR in addition to the X promoter–enhancer described earlier (Table II). The promoters for cellular proto-oncogenes, c-*jun* and c-*fos*, were also transactivated by trc-S. However, trc-S could transactivate only some homologous and heterologous viral sequences. For example, no transactivation or DNA binding was observed with the HBV core promoter–enhancer. Similarly, no activity was observed with SV40 early promoter–enhancer and HTLV-1 LTR. However, the enhancement of reporter

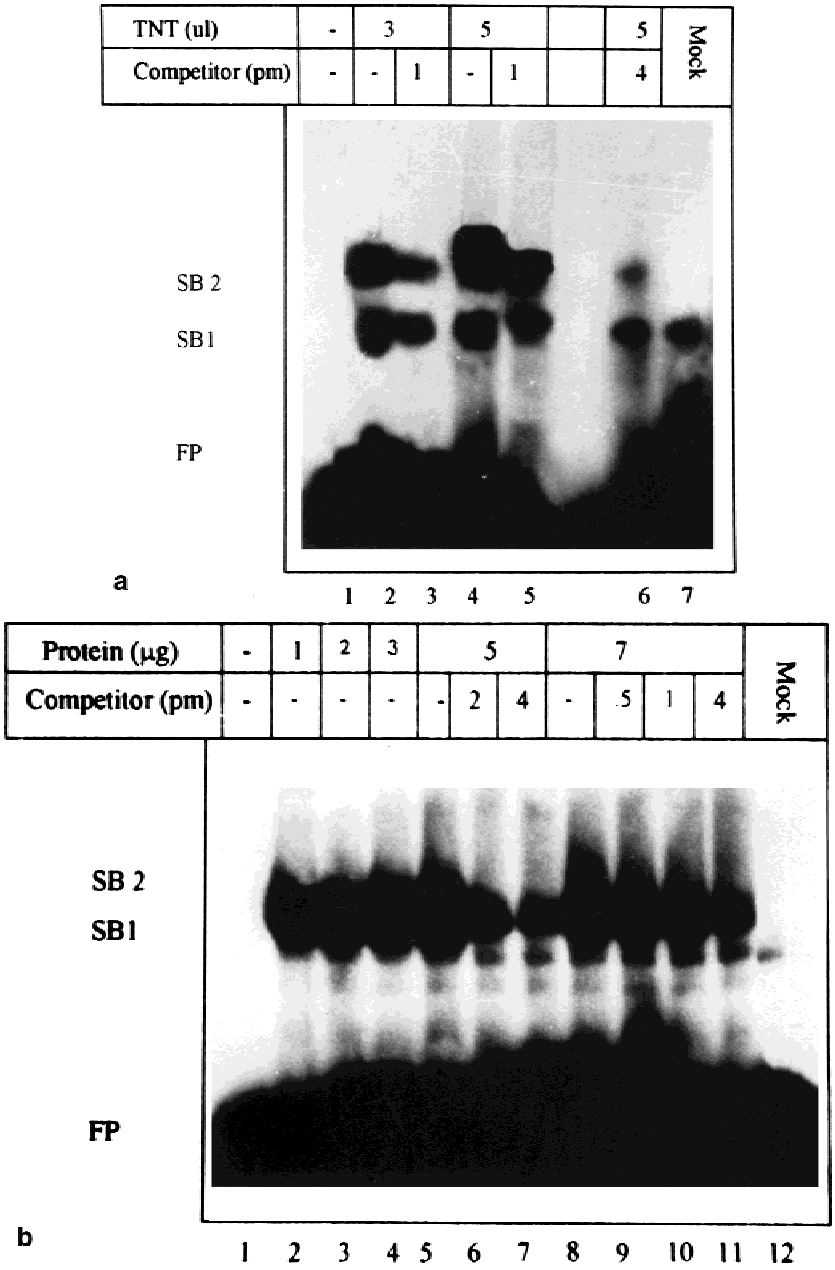

Fig. 7. **a:** Electrophoretic mobility shift assay showing the interaction of radiolabelled oligonucleotide representing the AP1 binding site with the in vitro–produced translated S protein (**lanes 2 and 4**). Specific unlabeled competitor (in pmol) was used as shown (**lanes 3, 5, and 6**). The mock lane (**lane 7**) represents the binding of AP1 oligonucleotide with the control nontranslated transcription-linked translation (TNT) lysate. FP, free probe (**lane 1**); SB1, shifted band 1; SB2, shifted band 2. (A blank in left between lanes 5 & 6) **b:** Gel retardation analysis showing the interaction between oligonucleotide representing the AP1 binding site and nuclear extracts prepared from pSG41-transfected HepG2 cells (**lanes 2, 3, 4, 5, and 8**). The amount of specific competitor is indicated at the top of the lane (**lanes 6, 7, 9, 10, and 11**). Nuclear extract prepared from pSG5-transfected HepG2 cells were used for mock (**lane 12**) binding reactions. FP, free probe (**lane 1**); SB1, shifted band 1; SB2, shifted band 2.

activity was only modest, however, indicating that trc-S is a weak transactivator.

Seto et al. [1988] showed that of the HBV gene products, only X protein transactivates HIV LTR in Jurkat cells. It was shown in this study that the carboxy terminal end of the HBV S region (nucleotides 426-855) does transactivate HIV-1 LTR in HepG2 cells, though modestly. The difference may be due to the different cell line used or to individual variations in the CAT assays. Conflicting results have also been found in terms of the capacity of HBx to transactivate certain regulatory sequences in a specific cell line. These results may stem from differences in the basal activity of reporter, since high basal activity would mask moderate levels of transactivation [Twu and Schloemer, 1987; Rossner, 1992]. Viral transactivators function either through direct sequence-specific DNA binding or by indirect means involving other cellular factors [Flint and

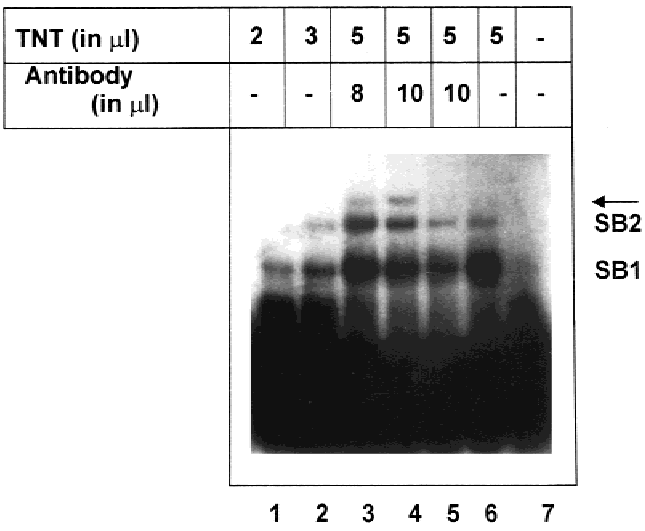

Fig. 8. Supershift assay was performed with in vitro–produced truncated S protein (trc-S) and γ-^{32}P-labelled TFIID-binding oligonucleotide in the presence of polyclonal antibodies against hepatitis B surface antigen (HbsAg). The radiolabelled probe was incubated either alone (**lane 7**) or with trc-S (**lanes 1, 2, and 6**). After complex formation, either 8 μl (**lane 3**) or 10 μl (**lane 4**) anti-HBsAg was added, respectively, to the reaction mix. The complex with the antibody is indicated with an arrow. **Lane 5** shows the interaction of the DNA–protein complex with serum negative for anti-HBsAg (negative control).

Shenk, 1989]. To elucidate the mechanism of transactivation by trc-S, we investigated the DNA-binding properties of this protein. We observed that the S transactivator protein (trc-S) bound to the HBV X promoter (Fig. 5a,b) but not to the core promoter. This finding correlates with the finding of this study that trc-S transactivates HBV X but not the core promoter.

Binding of the HBV X promoter was carried out with trc-S expressed both in vitro and in vivo. DNA–protein binding interactions of trc-S with HBV X promoter showed a single shifted band (SB). It also bound to the DNA-binding domains for TFIID and AP1 transcription factors (Figs. 6 and 7). Binding of TFIID and AP1 oligonucleotides with trc-S showed two retarded bands. The lower band (SB1) was also present in binding reactions of mock (pSG5)–transfected and nontransfected HepG2 cells as well as in binding with the control in vitro translation reaction. This may be the result of binding of AP1 and TFIID proteins present in HepG2 cells and in the translation mix. The second retarded band (SB2) was specific to the binding reactions between trc-S and the AP1 or TFIID binding domains. The specificity of the binding was confirmed with supershift assays (Fig. 8).

A dose-dependent response of trc-S was observed in terms of its transactivation as well as its binding properties. On increasing the amount of plasmid pSG41 in HepG2 cells by transfection, an initial increase in CAT activity was noted, which declined on increasing further the amount of transfecting plasmid pSG41 (Table II). The binding assays between trc-S and various promoters showed a similar concentration effect. There was an initial increase in the binding of DNA with an increase in the concentration of protein, which was followed by a decrease with further increase in the protein amount. This was true for HBV X promoter as well as for AP1 and TFIID binding domains. At higher concentrations, the HBV S transactivator might repress the target promoters, which might be due to squelching [Gill and Ptashne, 1988].

AP1 is a family of transcription factors formed by the products of proto-onocogenes c-*fos* and c-*jun* [Angel and Karin, 1991]. Transcription factor TFIID is a multiprotein complex composed of a TATA-box-binding subunit and several tightly associated factors [Delgeschlager et al., 1996]. RNA polymerase II requires TFIID to recognize the target promoter. The LTRs of retroviruses are conspicuous by the presence of various regulatory sequences that are important for viral transcription, host-cell tropism, and determination of pathogenetic capability [Major, 1990]. The promoter region in the LTRs of various retroviruses contain the TATA box (RSV LTR, HTLV-2 LTR, HIV-1 LTR) and the AP1 binding site (HIV-1 LTR). It was shown in our study that the carboxy terminal end of the S protein (trc-S) has the ability to bind to the AP1 and TFIID binding sites of these promoters. Thus, it is possible that the trc-S protein might have the ability to modify their activity.

Expression of the integrated S region might activate c-*jun*/c-*fos* in HCC, as shown by transient co-transfection assays in HepG2 cells. Activated oncogenes possibly play an important role in both viral and nonviral carcinogenesis. In HCC tissue with integrated HBV DNA, c-*jun* proto-oncogene expression was shown to be higher than in normal liver tissue [Twu et al., 1993]. Similarly c-*fos* expression has been found to be higher in HCC tissue [Tabor, 1994]. The truncated S protein produced in HCC may act as a transcriptional transactivator. It might switch on or modify the activity of cellular factors by binding to the AP1 and TFIID domains, thus leading to abnormal regulation of several genes, including some involved in growth regulation. The transactivator function of the HBV X protein is mediated primarily through interaction with cellular DNA-binding proteins [Maguire et al., 1991]. Such transactivators functioning via cellular factors may transactivate both cellular and viral sequences [Rossner, 1992]. The HBV trc-S protein binds directly to the transcription factor binding sites and also has the ability to activate several cellular and viral promoters.

The exact mechanism of HBV-associated neoplastic transformation of liver cells is not known. Progression of chronic liver disease, with associated liver cell necrosis, regeneration, and inflammation, definitely plays a major role, irrespective of its etiologic association. In the case of HBV-associated HCC, integrated viral genome or parts thereof are frequently detected [Hino et al., 1985; Zhou et al., 1987; Ramesh et al., 1994]. Several studies have found that different genes of HBV are capable of transactivation, for example, truncated X, pre-S2/S, pre-S1, and large surface region.

At the same time, alterations in gene functions of c-*myc* [Himeno et al., 1988], c-*fos*, c-*jun* [Varmus, 1984], and p53 [Bressac et al., 1990] have been observed in a limited number of cases associated with HCC. We show here that a truncated HBV S transactivator protein (trc-S) has the ability to activate both cellular and heterologus viral sequences and works through a DNA-binding mechanism. Therefore, it is possible that either one transactivator or the cumulative effect of several transactivators plays a role in hepatocarcinogenesis.

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